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Continuous production and recovery of recombinant Ca^{2+} binding receptor from HEK 293 cells using perfusion through a packed bed bioreactor

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Abstract

The extracellular domain of human parathyroid Ca^{2+} receptor was needed in order to study its structure and clinical application. The Ca^{2+} receptor is a unique member of the G protein-coupled receptor super-family, expressed in parathyroid and kidney cells where it has been shown to play a critical role in extracellular calcium homeostasis. The desired protein was produced by immobilizing the transformed HEK 293 cells in a packed-bed configuration using a 1.6 l (working volume) bioreactor equipped with a vertical mixing impeller assembly and an internal basket. The process included a propagation phase followed by a production phase. In the propagation phase, lasting approximately 160 h, the bed was perfused with a serum-containing medium, allowing the cells to grow at a constant growth rate to approximately 3×10^{10}. At this point the production phase was begun, replacing the medium with serum-free medium and continuing the perfusion process for additional 350 h. During this phase, the medium was pumped through the packed bed at a rate of 4–6 l per day, keeping the residual glucose concentration around 1 g l^{-1} and collecting and processing approximately 80 l of spent medium. This continuous perfusion method of the packed-bed bioreactor was compared to a repeated batch method in which existing medium was replenished when the glucose concentration was down to 1 g l^{-1}. Using this method, serum-free medium was replaced with serum containing medium a few times when a decline in the glucose consumption was observed. Though medium consumption and protein yield are similar in both methods (roughly 10 mg l^{-1}), there are differences related to the ease of operation and processing of the produced protein. The continuous perfusion operation was found to be preferable and was chosen as the production strategy.

Introduction

The extracellular domain of human parathyroid Ca^{2+} receptor was needed in order to study its structure and clinical application. The Ca^{2+} receptor is a unique member of the G protein-coupled receptor super-family, expressed in parathyroid and kidney cells where it has been shown to play a critical role in extracellular calcium homeostasis. The protein was cloned into HEK 293, a cell line derived from secondary embryonic kidney cells transformed with sheared fragments of adenovirus (Graham et al., 1977). These cells produced the protein constitutively and secreted it to the medium. These anchorage-dependent cells (Graham, 1986) are easily transformed to produce human heterologous proteins (Berg et al., 1993).

Several methods are available for the large-scale growth of anchorage-dependent cells. Cells can grow on micro carriers in a stirred-tank reactor, on macroporous micro carriers in a fluidized bed bioreactor, on a stationary matrix in a packed-bed bioreactor, on hollow fibers or on flat sheets. When dealing with anchorage-dependent cells, medium replacement is the accepted method to sustain growth and protein production by maintaining a relatively constant supply of growth factors such as carbon source, amino

acids and vitamins, while lowering the concentration of inhibitory metabolites such as lactic acid and ammonia.

The packed-bed bioreactor is an efficient and convenient system for the production of extracellular biomolecules from trapped or immobilized microorganisms. In the last few years, this method has also gained popularity for the production of proteins from anchorage-dependent as well as from anchorage-independent mammalian cells. The development of various packed bed matrices, such as porous ceramic beads (Park and Stephanopoulos, 1993), glass fibers (Chiou et al., 1991), porous glass beads (Racher and Griffiths, 1993; Yoshida et al., 1997), cylinder shaped sintered glass carriers (Rzeski et al., 1993) and polyester disks (Racher et al., 1995), and the development of a bioreactor configuration suitable for fragile cells, are the main reasons for the growing interest in this production system. Cell concentration between 10^7 and 5×10^8 ml^{-1} of packed bed have been obtained during production of proteins such as insulin (Park and Stephanopoulos, 1993), γ-interferon (Chiou et al., 1991), interluken-6 (Yoshida et al., 1997) and monoclonal antibodies (Racher and Griffiths, 1993) from various cells such as AtT-20, CHO, BHK and hybridomas, respectively.

Packed-bed bioreactors are appropriate for production of extracellular proteins because they are easy to use, they allow cells to grow to high densities due to the large surface area, and they are suitable for medium replacement operation. On the other hand, compared to fluidized bed or stirred tank reactors, the packed bed is inhomogeneous and the sampling of cells is not possible. In the present work, we report on the use of a packed bed made of polyester disks for the production of human parathyroid Ca^{2+} binding protein from human embryonic kidney cells (HEK 293).

To simplify the recovery and purification of the protein, the production is done in serum-free medium and, therefore, the overall process is done in two phases (Shiloach et al., 1996). In the propagation phase the cells are grown in serum-containing medium; in the production phase, the desired protein is produced and secreted to the serum-free medium. During each phase, the medium needs to be replaced to maintain growth and production. Medium replacement (perfusion) can be performed in several ways: if small variations in metabolite concentration affect cellular metabolism and protein biosynthesis, continuous perfusion is the optimal method. However, if cellular metabolism and protein biosynthesis are not affected by changes in nutrient concentration and metabolite formation, batch or semi batch replacement of medium may be performed.

The extracellular domain of human parathyroid Ca^{2+} receptor was produced in a packed-bed bioreactor using continuous medium replacement or semi-batch replacement. The comparison between these two methods, the effect of serum-free medium on cell growth, and the specific utilization of the media are described.

Materials and methods

Cell lines and media

Human embryonic kidney cells (HEK 293), transformed to produce the extracellular domain of the human parathyroid Ca^{2+} receptor (Goldsmith et al., 1999) were obtained from NPS Pharmaceuticals (U.S.A.), and were routinely passaged in tissue culture flasks (Costar Corporation, U.S.A.) using DMEM with glutamine (Biofluids Inc., U.S.A.) supplemented with 10% fetal bovine serum (Biofluids), and 200 U ml^{-1} hygromycin B (Calbiochem Corporation, U.S.A.) which was used as the selective marker. Propagation medium consisted of DMEM supplemented with 10% fetal bovine serum, 200 U ml^{-1} Hygromycin B and 0.1% pluronic F-68 (Gibco, U.S.A.). Production medium consisted of DMEM without phenol red (phenol red interferes with the purification process, data not shown), containing serum-free supplement specifically made for HEK 293 (Kemp Biotechnologies, U.S.A.) (Shiloach et al., 1996), 2 mM glutamine (Gibco), 200 U ml^{-1} Hygromycin B and 0.1% pluoronic F-68.

Reactor system and cultivation conditions

Perfusion process

Cells grown in tissue culture flasks were transferred to a 2.2 l bioreactor (1.6 l working volume) equipped with an internal retention device (basket) and a vertical mixing system (Celligen Plus^{TM}; New Brunswick Scientific, U.S.A.). The basket contained 60 g of FibraCel^{TM} disks (Bibby Sterlin, U.K.) made of polyester non woven fabric laminated to a polypropylene screen, treated and pre-coated with poly-D-lysin. The dissolved oxygen concentration (DO) was kept at 50% air saturation by sparging with air and or oxygen. The

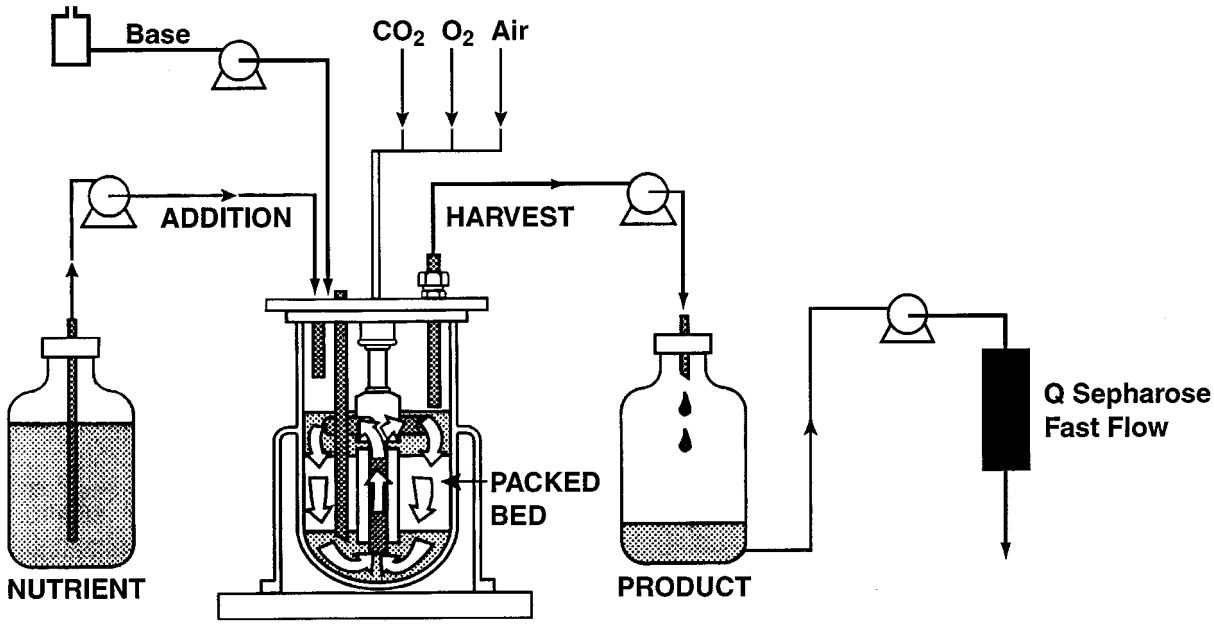

Figure 1. Overall production scheme for a perfused packed bed process.

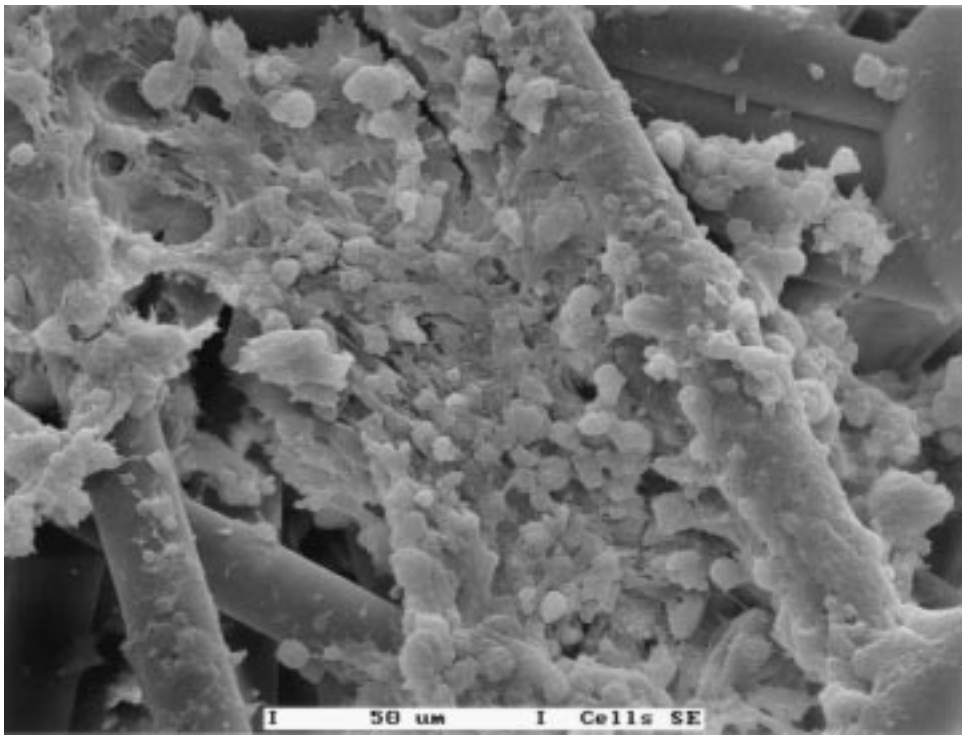

Figure 2. Electron scanning micrograph of glutaraldehyde fixed polyester disks from a terminated packed bed process carrying adherent cells.

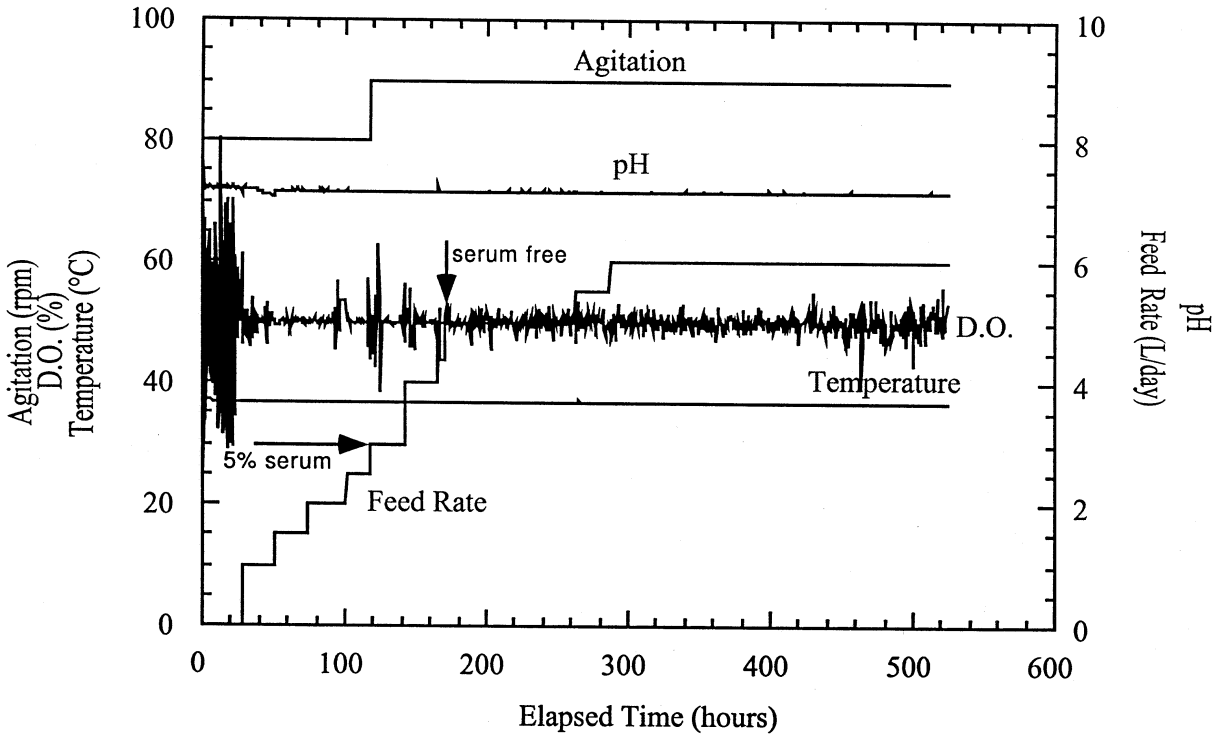

Figure 3. Fermentation parameters during the perfusion process through the packed bed bioreactor. Dissolved oxygen (DO) was kept at 50% air saturation, temperature at 37 °C, pH at 7.0, initial agitation rate was 80 RPM and it was increased to 90 after 120 h, the initial feeding rate was 1 l per day and it increased to 6 l per day.

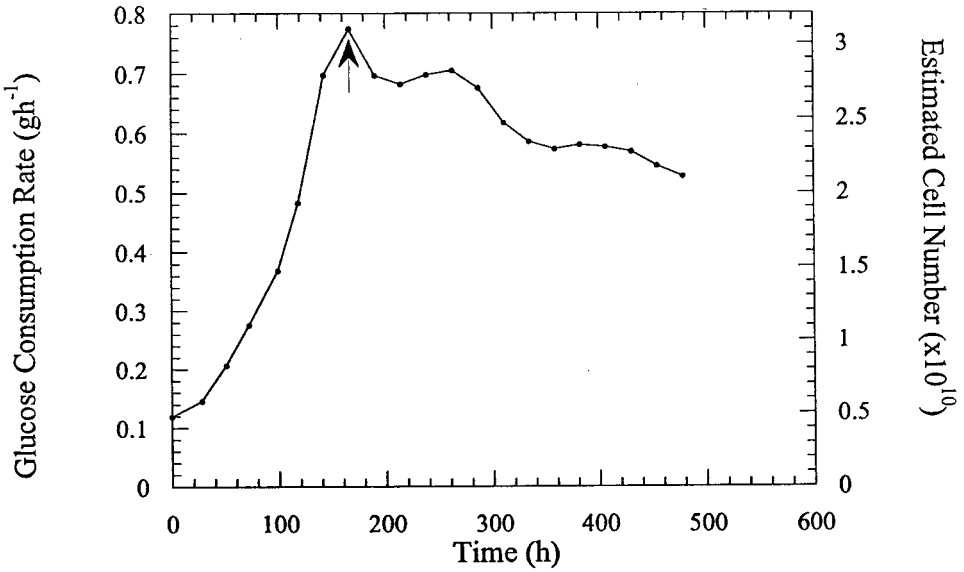

Figure 4. Glucose consumption rate and estimated cell number for a perfused packed bed process; arrow indicates change from the propagation phase to the production phase.

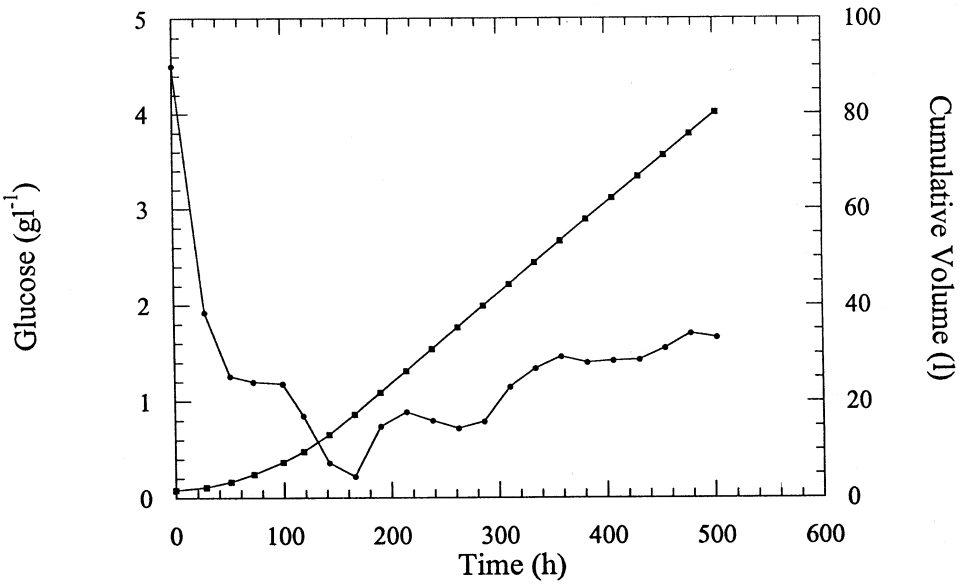

Figure 5. Glucose concentration and cumulative volume of media used during perfused packed bed process.

pH was kept at 7.0 by sparging carbon dioxide or by adding 0.2 M $NaHCO_3$ directly to the bioreactor. The initial agitation was 80 RPM and it was increased to 90 RPM. The temperature was kept at 37 °C.

During the propagation phase, the reactor was operated in a batch mode until the glucose concentration reached 1.0 g l^{-1}, at which point the packed bed was perfused with propagation medium to keep the glucose at that level. After 120 h the serum concentration in this medium (Figure 3) was reduced to 5%. The production phase started when the propagation medium was substituted with serum-free medium which was perfused through the packed bed. Spent medium was collected into an intermediate reservoir from which it was pumped onto an ion-exchange column (described below). The overall production layout is shown in Figure 1. Figure 2 is a scanning electron micrograph of glutaraldehyde fixed polyester disks showing adherent cells, and the production process is described in Figure 3.

Repeated batch process

Cells grown in tissue culture flasks were transferred to a 5.0 l (working volume) Celligen Plus™ fermentor containing 60 g of Fibra-Cel™. Fermentation parameters were the same as in the perfusion process. The reactor was operated in a batch mode until the glucose concentration reached 1.0 g l^{-1}, at which point the medium in the fermentor was replaced with fresh medium by first pumping out the old medium, and then pumping in the fresh medium. During the production phase, propagation medium was substituted with production medium, and the spent medium was passed through an ion-exchange column (described below).

Protein recovery

A Q Sepharose Fast Flow column (Amersham Pharmacia Biotech) was equilibrated with PBS. The spent production media, containing the Ca^{2+} binding protein was pumped through the column at a ratio of 1 l per 10 ml of packed resin. The protein was then eluted with 1.5 column volumes of PBS containing 1.0 M NaCl. Further purification of the protein was done using a lectin column.

Analysis of samples

Glucose and lactate concentrations were determined using a YSI analyzer (Yellow Springs Instruments, U.S.A.).

The production of the Ca^{2+} binding receptor was detected by immunoblot: medium samples collected during the propagation and the production phases were concentrated 10 times with Centriprep 10 spin concentrators (Amicon Inc., U.S.A.) and electrophoresed on an 8–16% SDS acrylamide gel (Novex, U.S.A.) under reducing conditions. The proteins were transferred to a nitrocellulose filters at 30 V for 1 h. The filters were incubated with a primary antibody, (rabbit) for 1 h, washed with PBS containing 0.1% Tween-20, and

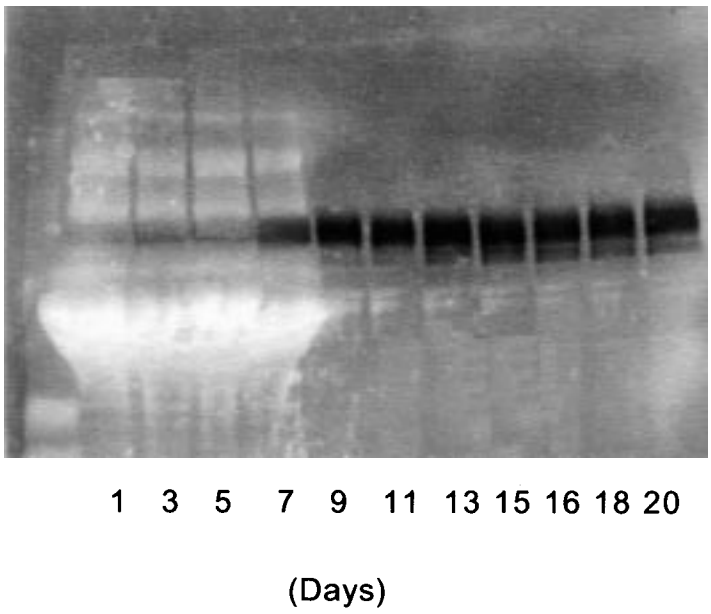

Figure 6. Western blot from samples obtained at different times during a perfused packed bed process; switch from serum-containing media to serum-free medium was done at day 7.

incubated with a secondary antibody (goat anti rabbit IgG alkaline phosphatase conjugate), (Kirkegaard and Perry, U.S.A.), for 1 h. The filters were washed with PBS-Tween and developed using a BCIP/NBT phosphatase substrate solution, and the blots were scanned using a snap Scan 600.

Quantitative determination of the produced protein were done after the Q Sepharose FF (Amersham Pharmacia Biotech) by adsorption to nitrocellulose using a filtration manifold (Minifold SRC-96, Schleicher and Schuell). The protein was detected as described above and quantitated using the NIH image 1.62 software.

Fibra Cel^{TM} disks obtained from terminated culture were fixed with 2% glutaraldehyde and dehydrated with ethanol. The samples were then analyzed with a scanning electron microscope by Advanced Research Instruments Corporation (U.S.A.) at 500× magnification.

Specific glucose consumption rate

The specific glucose consumption rate of HEK 293 cells was determined in tissue culture flasks. Ten T-25s (Costar) were inoculated with approximately 6.5×10^5 cells in 5 ml complete propagation medium and incubated at 37 °C, every day thereafter, a flask was removed, the glucose concentration was determined, the cells were gently scraped, the viable cell number was determined, and the specific glucose consumption rate was calculated. The average value was 25 μg per 10^6 cells per hour. This value was used to determine cell numbers when using both the continuous perfusion method and the repeated batch method, whether the medium included serum or not.

Results

Continuous perfusion process

The continuous perfusion process is described in Figures 3–5. Figure 3 describes process parameters: feed (media replacement) rate, dissolved oxygen concentration (DO), agitation rate, pH and temperature. The total cell number, based on glucose consumption rate, is shown in Figure 4; glucose concentration and the amount of medium perfused through the bioreactor during the overall process (80 l in 500 h) are shown in Figure 5.

The 1.6 l (working volume) bioreactor containing 60 g of Fibra Cel^{TM} disks was inoculated with 7×10^9 cells. During the propagation phase, lasting approximately 160 h, the bed was perfused initially with medium containing 10% serum, and after 120 h, when the cell number was approximately 2×10^{10}, the serum concentration was lowered to 5%. After additional 40 h, when the total cell number reached 3×10^{10}, the production phase started and serum-free

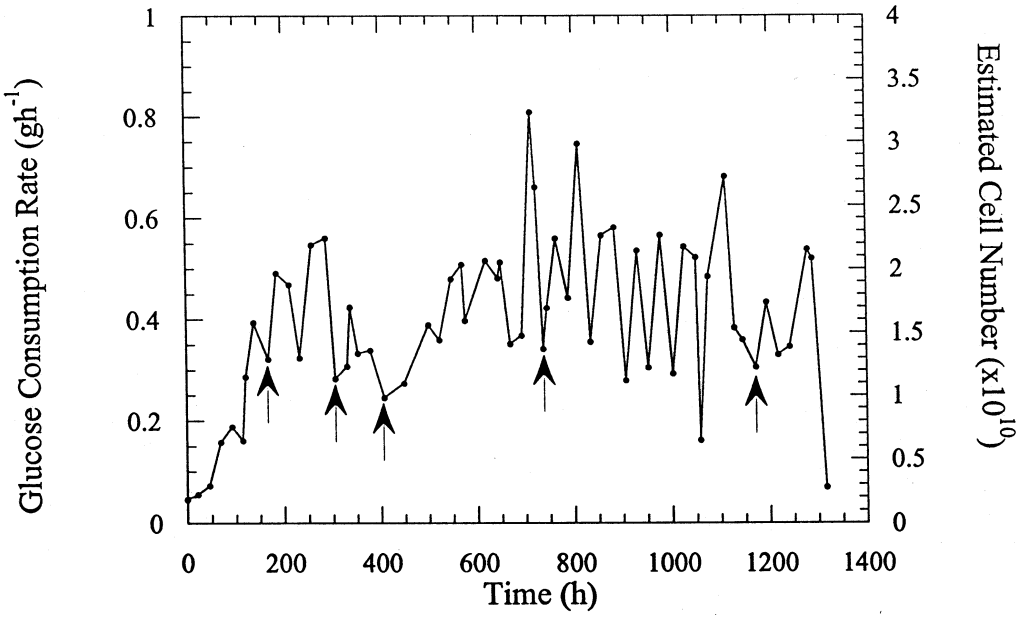

Figure 7. Glucose consumption rate and estimated cell number for a repeated batch packed bed process; arrows indicate when media containing serum was introduced during the process.

medium replaced the serum containing medium. To keep the glucose concentration at 1.0 g l^{-1}, the initial perfusion rate was 1.0 l per day and it increased gradually to a final rate of 6 l per day. The initial agitation speed was 80 rpm and it was increased to 90 rpm after 120 h. During the propagation phase, the cells grew at a constant growth rate of 0.0126 h^{-1} (Figure 4) as estimated from specific glucose consumption rate of 25 $\mu g/10^6$ cells h^{-1} (see the Materials and Methods section). During the production phase, lasting approximately 350 h, the cell number declined linearly at a rate of 2.8×10^7 cells per hour to 2×10^{10} cells.

A western blot of the Ca^{2+} binding protein produced during the process is seen in Figure 6; the protein level seems to be constant throughout the entire production phase. The transition from serum-containing medium to serum-free medium is seen clearly by the lower amount of total protein from the ninth day on. As seen in Figure 1, the Ca^{2+} binding protein was continuously recovered by pumping the spent media directly on the anion-exchanger column (Q Sepharose Fast Flow). Approximately 10 mg per liter of protein was recovered.

Repeated batch process

The repeated batch process is described in Figures 7 and 8. A five-liter packed-bed bioreactor, containing 60 g of Fibra Cel^{TM} disks, was inoculated with 2.5×10^9 cells. During the propagation phase which lasted approximately 250 h, the cell number increased to about 2.5×10^{10}. At that time, the serum-containing medium was replaced with serum-free medium and the production phase started. The medium in the reactor was replaced when the glucose concentration fell below 1.0 g l^{-1}. Up to 6 replacements of medium were done before a decrease in glucose consumption rate was observed. At this point the serum-free medium was replaced with serum-containing medium for 24 h followed by a return to serum-free medium. Using this cycling strategy, the process was maintained for a period of 44 days, during which the glucose consumption rate fluctuated between 0.3 and 0.6 g h^{-1} (Figure 7); this fluctuation was attributed to the changes in glucose concentration (Figure 8). The process was terminated when the glucose consumption rate could not be restored by the cycling strategy. A loss of adherence by a large portion of the cell population was observed and is probably the cause the deterioration in glucose consumption.

Discussion and conclusions

Extracellular recombinant proteins can be produced from anchorage-dependent mammalian cells in sev-

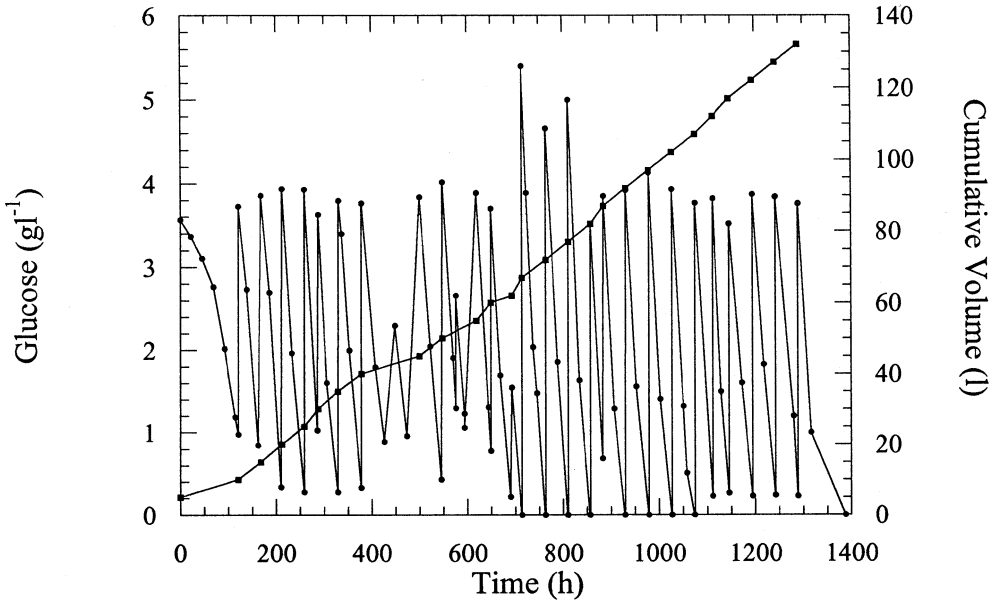

Figure 8. Glucose concentration and cumulative volume of media used for a repeated batch packed bed process.

eral configurations. Among these are micro-carriers in stirred-tank reactors, macroporous micro carriers in fludized-bed bioreactors, various adsorbent materials in packed bed reactors, hollow fiber reactors and flat surface arrangements (Sambanis and Hu, 1993). As was pointed out in previous work (Shiloach et al., 1996), the packed-bed bioreactor is a convenient and efficient arrangement for producing extracellular proteins from both anchorage-dependent and anchorage-independent cells. It provides large surface area in a relatively small volume, and it allows easy medium replacement and continuous perfusion. The dissolved oxygen concentration and pH are controlled by direct aeration of the vertical mixing bioreactor, an arrangement that is less complicated than the one in continuous flow across flat surfaces or in the hollow fiber layout. In addition, because the cells are immobilized, it is much easier to replace the medium in a packed-bed bioreactor than it is in a stirred-tank bioreactor containing micro carriers.

Extracellular proteins from mammalian cells are preferably produced in serum-free medium to simplify the recovery and purification, and to ensure consistent quality of the product. Therefore, after achieving the proper biomass concentration using serum-containing medium, the medium is replaced with serum-free one. Because this medium cannot support cell growth, the cell number declines approximately 30% in 400 h of continuous perfusion, suggesting that, very likely, serum-containing medium is needed periodically to restore the activity or the viability of the adherent cells.

The continuous perfusion process was performed in a 1.6 l (working volume) bioreactor with maximum perfusion rate of 6 l per day keeping the glucose concentration at 1 g l^{-1}. An alternative process was conducted in a 5 l packed-bed bioreactor containing the same amount of adsorbent material, using repeated batch mode to replace 100% of the medium in the bioreactor once a day.

The repeated batch mode is easy to implement because it does not require continuous pumping and it allows for easy alternation between serum-containing medium and serum-free medium. In this mode, the glucose concentration fluctuates between 4.0 g l^{-1} and 0.2 g l^{-1}, which may affect the glucose consumption rate of the cells, as can be seen in Figure 7. But these fluctuations in the glucose concentration do not have an effect on the overall glucose consumption and on the amount of the protein produced (Shiloach et al., 1996).

Because it is impossible to count the cells directly, the cell number is estimated from glucose consumption values. This estimate is based on the assumption that the glucose consumption rate does not depend on method of perfusion (continuous or repeated batch) and does not depend on the glucose concentration nor on serum concentration. Even though the amount of carriers in the two methods was the same, the total cell

number in the repeated batch was lower than the total cell number in the perfused system, around 2×10^{10} compared with 3×10^{10}. The amount of medium used was higher in the perfused system, approximately 1.3 l per day per 1×10^{10} cells, compared with 1.1 l per day per 1×10^{10} cells. Very likely, the reason for the higher concentration in the perfusion system is the fact that the glucose concentration was kept at 1.0 g l^{-1} while in the repeated batch it was allowed to go below 1.0 g l^{-1}. The secreted protein concentration was similar in both methods, roughly 10 mg l^{-1}, indicating that the production is dependent on the amount of nutrients used and not on the medium replacement strategy.

In our opinion, the continuous operation mode is preferable to the repeated batch mode because it keeps the cells in a homeostatic environment much like that experienced in vivo (Feder, 1988). In addition, pumping the spent medium directly on an ion-exchange column simplifies the recovery process and improves product stability. The observed decline in the cell number may be eliminated by devising a strategy that incorporates periodic changes between serum-free medium and serum-containing medium, or the development of a better serum-free medium.

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